Center for Veterinary Biologics
and
National Veterinary Services Laboratories
Testing Protocol

Supplemental Assay Method for Titration of Feline *Chlamydia psittaci* in Embryonated Chicken Eggs

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1. Introduction

1.1 Background

This Supplemental Assay Method (SAM) is a titration method for assaying live chicken-embryo-adapted feline *Chlamydia psittaci* vaccines for potency. The method uses embryonated chicken eggs as the indicator host system. This assay is used to demonstrate the presence of live chlamydia and to determine the titer of a vaccine serial by the chicken embryo death pattern 10 days after inoculation.

1.2 Keywords

Feline *Chlamydia psittaci*, potency test, chicken embryo titration, $CEID_{50}$

2. Materials

2.1 Equipment/instrumentation

2.1.1 Egg incubator,[1] 36^0 ! 2^0C, humidified

2.1.2 Cabinet,[2] laboratory biosafety level-2

2.1.3 Water bath,[3] 36^0 ! 2^0C

2.1.4 Vortex mixer[4]

2.1.5 Micropipettor,[5] 1000 µl, and tips[6]

1 [1]Jamesway Model No. 252, Midwest Incubators Sales and Service, 1650 Washington St.,
2 P.O. Box 88, Carlyle, IL 62231 or equivalent
3 [2]Model No. NU-407FM-400, NuAire, Inc., 2100 Fernbrook Ln., Plymouth, MN 55441 or equivalent
4 [3]Cat. No. 15-461-10, Fisher Scientific Corp., 2000 Park Ln., Pittsburg, PA 15275 or equivalent
5 [4]Model G-560, Scientific Industries, Inc., 700 Orville Dr., Bohemia, NY 11716 or equivalent
6 [5]Cat. No. P-1000, Rainin Instrument Co., Box 4026, Mack Rd., Woburn, MA 01888 or equivalent
7 [6]Cat. No. YE-3R, Analytic Lab Accessories, P.O. Box 345, Rockville Center, NY 11571 or equivalent

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2.2 Reagents/supplies

2.2.1 Chlamydia Reference,[7] Cello strain

2.2.2 Embryonated chicken eggs,[8] 6-7 day, specific pathogen free, in accordance with Code of Federal Regulations, Title 9 (9 CFR)

2.2.3 7.5% Sodium Bicarbonate

2.2.3.1 7.5 g sodium bicarbonate

2.2.3.2 Q.S. to 100 ml with deionized water

2.2.3.3 Sterilize by autoclaving at $121^0 \pm 2^0$C, 15 psi for 30 ± 10 min.

2.2.3.4 Store at $4^0 \pm 2^0$C.

2.2.4 1% Phenol Red

2.2.4.1 1.0 g phenol red[9]

2.2.4.2 Q.S. to 100 ml with DW.

2.2.4.3 Store at $4^0 \pm 2^0$C.

2.2.5 Sucrose phosphate buffer of Bovarnick (Chlamydia Diluent)

2.2.5.1 74.6 g sucrose ($C_{12}H_{22}0_{11}$)[10]

2.2.5.2 0.42 g potassium phosphate, monobasic, anhydrous (KH_2P0_4)[11]

2.2.5.3 1.25 g potassium phosphate, dibasic, anhydrous (K_2HP0_4)[12]

2.2.5.4 0.92 g monosodium glutamate

2.2.5.5 1.0 ml gentamicin[14]

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8 [7]Available upon request from the Center for Veterinary Biologics-Laboratory (CVB-L), 9 P.O. Box 844, Ames, IA 50010 or equivalent
10[8]SPAFAS, 190 Route 165, Preston, CT 06365 or equivalent
11[9]Cat. No. T265-03, J.T. Baker, Inc., 222 Red School Ln., Phillipsburg, NJ 08865 or equivalent
12[10]Cat. No. S5-500, Fisher Scientific Corp. or equivalent
13[11]Cat. No. 3246-01, J.T. Baker, Inc. or equivalent
14[12]Cat. No. 3252-01, J.T. Baker, Inc. or equivalent
15[13]Cat. No. G-1626, Sigma Chemical Co., P.O. Box 14508, St. Louis, MO 63178 or equivalent
16[14]Gentocin® solution, Cat. No. 0061-0464-04, Schering-Plough Animal Health Corp., 172015 Galloping Hill Rd., Kenilworth, NJ 07033-1300 or equivalent

2.2.5.6 1 ml 1% Phenol Red

2.2.5.7 Dissolve ingredients in DW to make 1 L.

2.2.5.8 Filter through a 0.22-µm filter.[15]

2.2.5.9 Adjust pH to 7.0-7.2 with 7.5% Sodium Bicarbonate.

2.2.5.10 Store at $4^0 \pm 2^0$C.

2.2.6 Ethanol Solution, 70%

2.2.6.1 718 ml ethanol, 95%[16]

2.2.6.2 282 ml DW

2.2.6.3 Store at room temperature (RT) ($23^0 \pm 2^0$C).

2.2.7 Tincture of Iodine, 2%

2.2.7.1 2 g iodine[17]

2.2.7.2 100 ml 70% Ethanol Solution

2.2.7.3 Store at RT.

2.2.8 Egg-candling light[18]

2.2.9 Etcher/engraver,[19] electric

2.2.10 Needles, 22 ga x 1½ in[20] and 18 ga x 1½ in[21]

2.2.11 Syringe,[22] 1 ml tuberculin

2.2.12 Duco cement[23]

18[15]Cat. No. 12122, Gelman Sciences, 600 S. Wagner Rd., Ann Arbor, MI 48106 or equivalent
19[16]Cat. No. 7018-08, Mallinckrodt, 222 Red School Lane, Philipsburg, NJ 08865 or equivalent
20[17] Product No. 137-500, Fisher Chemical, Fisher Scientific, 50 Faden Road, Springfield, NJ 07081 or equivalent.
21[18]Model No. 350, Reichert Scientific Instruments, P.O. Box 123, Buffalo, NY 14240 or 22equivalent
23[19]Vibro-graver, Acme Burgess, Inc., Grayslake, IL 60030 or equivalent
24[20]Cat. No. 5159, Becton Dickinson & Co., 1 Becton Dr., Franklin Lakes, NJ 07417-1884 or equivalent
25[21]Cat. No. 305196, Becton Dickinson & Co. or equivalent
26[22]Cat. No. 309602, Becton Dickinson & Co. or equivalent
27[23]Cat. No. 6243, DevCon Consumer Products, Des Plaines, IL 60018 or equivalent

2.2.13 Polystyrene tubes,[24] 17 x 100 mm

2.2.14 Pipette-aid[25]

2.2.15 Pipettes, 10 ml[26]

3. Preparation for the test

3.1 Personnel qualifications/training

Personnel shall have experience in the propagation and maintenance of chlamydial agents and inoculation techniques of embryonated chicken eggs. Personnel shall be proficient with chlamydia titration techniques using embryonated chicken eggs.

3.2 Preparation of equipment/instrumentation

On the day of test initiation, set a water bath at $36^0 \pm 2^0$C.

3.3 Preparation of reagents/control procedures

3.3.1 Preparation of eggs

3.3.1.1 On the day of test initiation, using an egg-candling light, check each egg for viability, proper embryo growth, and integrity of the egg shell. Appropriately dispose of eggs which do not meet these criteria.

3.3.1.2 Label each egg by writing a number with a pencil just below the base line of the air sac. Numbered eggs are placed in double layer cardboard flats.

3.3.1.3 Disinfect the air sac end by swabbing with 2% Tincture of Iodine. Allow to air dry.

3.3.1.4 Drill a small hole through the disinfected egg shell using an electric etcher/engraver.

28[24]Falcon® Cat. No. 2057, Becton Dickinson Labware, 2 Bridgewater Ln., Lincoln Park, NJ 07035 or equivalent
29[25]Cat. No. 183, Drummond Scientific Co., 500 Pkwy., Broomall, PA 19008 or equivalent
30[26]Falcon® Cat. No. 7530, Becton Dickinson Labware or equivalent

3.3.1.5 Retain 5 uninoculated viable embryonated chicken eggs to serve as controls and to monitor nonspecific embryo death.

3.3.2 Preparation of Chlamydia Reference Control

3.3.2.1 On the day of test initiation, rapidly thaw a vial of Chlamydia Reference in a $36^0 \pm 2^0$C water bath.

3.3.2.2 With a 10-ml pipette, dispense 4.5 ml Chlamydia Diluent into each of an appropriate number of 17 x 100-mm polystyrene tubes. Label each tube, bracketing the expected Chlamydia Reference endpoint titer specified in the CVB-L Reference and Reagent sheet (e.g., label 7 tubes from 10^{-1} through 10^{-7}, respectively).

3.3.2.3 With a micropipettor, transfer 500 µl of the Chlamydia Reference to the tube labeled 10^{-1}; mix by vortexing.

3.3.2.4 Using a new pipette tip, transfer 500 µl from the 10^{-1}-labeled tube (**Section 3.3.2.3)** to the 10^{-2} tube; mix by vortexing.

3.3.2.5 Repeat **Section 3.3.2.4** for each of the subsequent dilutions until the tenfold dilution series is completed.

3.4 Preparation of the sample

3.4.1 The initial test of a Test Serial will be with a single vial (a single sample from 1 vial). On the day of inoculation, using a sterile 1.0-ml syringe and an 18-ga x 1½-in needle, rehydrate a vial of the Test Serial with the provided diluent by transferring 1.0 ml for a 1-ml-dose vaccine, 0.5 ml for a ½-ml-dose vaccine, etc., into the vial containing the lyophilized Test Serial; mix by vortexing. Incubate for 15 ± 5 min at RT.

3.4.2 Multifraction Test Serials containing feline rhinotracheitis virus (FRV), feline calicivirus (FCV), or feline panleukopenia virus (FPV) are prepared the same as chlamydia single-fraction Test Serials. (FRV, FCV, and FPV do not replicate in embryonated chicken eggs.)

3.4.3 Using the same method described for diluting the Chlamydia Reference Control in **Section 3.3.2.2** through **Section 3.3.2.5**, prepare an appropriate number of tenfold serial dilutions of the reconstituted Test Serial to bracket its expected endpoint titer specified in the Animal and Plant Health Inspection Service (APHIS) filed Outline of Production.

4. Performance of the test

4.1 Mix the dilution tubes by vortexing, and aspirate 0.5 ml of the highest (most dilute) tenfold dilution of the Test Serial with a 1-ml syringe and 22-ga x 1½-in needle. Holding the syringe vertically, insert the needle to a depth of 1.25 ± 0.25 in. Inoculate 100 µl of the Test Serial into the yolk sac of each of 5 eggs/dilution.

4.2 With the same syringe, aspirate an equal volume of the vortexed next lower Test Serial dilution and inoculate the yolk sacs of 5 more eggs.

4.3 Inoculate the remaining dilutions. Separate syringes are not necessary between dilutions in a dilution series when dispensing from the most dilute to the most concentrated within that series but are required between series.

4.4 In a similar manner, inoculate 5 eggs/dilution of the Chlamydia Reference Control (dilutions 10^{-7} through 10^{-4}, **Section 3.3.2.2**).

4.5 Seal the inoculation hole of all eggs with Duco cement and allow to air dry 5 ± 2 min.

4.6 Return all inoculated embryonated chicken eggs to the egg incubator.

4.7 On the third day postinoculation (DPI), candle the inoculated and uninoculated control eggs. Identify, record day of death, and appropriately discard dead embryonated chicken eggs. Deaths occurring prior to 4 DPI are regarded as nonspecific deaths.

4.8 Candle the embryonated chicken eggs daily through 11 ± 1 DPI. Identify, record day of death, and appropriately discard dead embryonated chicken eggs. These deaths are regarded as specific and are used to calculate the titer of the Test Serial.

4.9 Calculate the *C. psittaci* endpoints of the Test Serial and the Chlamydia Reference Control using the Spearman and Kärber method as commonly modified. The titers are expressed as $\log_{10}$ 50% chicken embryo infective dose ($CEID_{50}$) per ml.

Example:

10^{-4} dilution of Test Serial = 5/5 embryos dead
10^{-5} dilution of Test Serial = 5/5 embryos dead
10^{-6} dilution of Test Serial = 2/5 embryos dead
10^{-7} dilution of Test Serial = 0/5 embryos dead

Test dose titer = (X - [d/2 + s] where:

X = $\log_{10}$ of lowest dilution (4)
d = $\log_{10}$ of dilution factor (1)
s = sum of proportion of dead chick embryos

$\frac{(5+5+2)}{5} = \frac{12}{5} = 2.4$

Test dose titer = (4 - 1/2) + (1 * 2.4) = 5.9

Adjust the titer to the Test Serial dose size by adding the $\log_{10}$ of the reciprocal of Inoculation Dose divided by the Test Serial Dose where:

Inoculation Dose = amount of diluted Test Serial with which each chicken embryo is inoculated

Test Serial Dose = manufacturer's recommended vaccination dose

Example:

Chlamydia endpoint= 5.9

0.1-ml dose = $\frac{1}{10}$= 1.0
Total = 6.9 log

Titer of the Test Serial is $10^{6.9}$ $CEID_{50}$

5. Interpretation of the test results

5.1 For a valid assay

5.1.1 All 5 of the uninoculated embryonated chicken eggs must be viable at the end of the test.

5.1.2 At least 4 eggs/dilution must be viable 4 DPI for each Test Serial and the Chlamydia Reference Control.

5.1.3 The calculated $CEID_{50}$ titer of the Chlamydia Reference Control must fall within plus or minus 2 standard deviations (± 2 SD) of its mean titer, as established by a minimum of 10 previous chlamydia titrations.

5.1.4 If the validity requirements are not met, then the assay is considered a **NO TEST** and can be retested without prejudice.

5.2 In a valid test, if the titer of the Test Serial is equal to or greater than the titer specified in an APHIS filed Outline of Production, the Test Serial is considered **SATISFACTORY**.

5.3 In a valid test, if the titer of the Test Serial is lower than the titer specified in an APHIS filed Outline of Production, the Test Serial shall be retested in accordance with 9 CFR, Part 113.8(b).

6. Report of test results

Report results as $CEID_{50}$ per dose.

7. References

7.1 Code of Federal Regulations, Title 9, Part 113.71, U.S. Government Printing Office, Washington, DC, 2000.

7.2 Cottral, GE (Ed.). *Manual of standardized methods for veterinary microbiology*. Comstock Publishing Associates, Ithaca, NY, 1978, pg. 731.

7.3 Finney, DJ. *Statistical method in biological assay*, 3rd edition. Griffin, London, 1978, pg. 508.

7.4 Richmond JY, McKinney RW (Eds.). *Biosafety in microbiological and biomedical laboratories*. U.S. Department of Health and Human Services, U.S. Government Printing Office, Washington, DC, 1993, pg. 177.

7.5 Storz, J. *Chlamydia and chlamydia-induced diseases*. Charles C. Thomas Publishing Company, Springfield, IL, 1971, pg. 358.

8. Summary of revisions

This is the revised version of the first draft written on 7/28/95. This document was rewritten to meet the current NVSL/CVB QA requirements, to clarify practices currently in use at the CVB-L for testing live *Chlamydia psittaci* vaccines, and to reflect the change in Chlamydia Diluent in the procedure from the superseded protocol.